Toxicity of engineered copper (Cu(0)) nanoparticles to the green alga *Chlamydomonas reinhardtii*

Emanuel Müller[1,2], Renata Behra[1,2], Laura Sigg[1,2*]

[1] *Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland*

[2] *ETH Zürich, Swiss Federal Institute of Technology, Institute of Biogeochemistry and Pollutant Dynamics, 8092 Zürich, Switzerland*

Abstract

Toxicity of carbon coated copper nanoparticles (CuNP) to the unicellular green alga *Chlamydomonas reinhardtii* was investigated and compared with effects of dissolved Cu^{2+}. The CuNP with original size of 6 – 7 nm rapidly agglomerated in the medium to average particle sizes of 140 – 200 nm. Dissolved Cu from CuNP increased over 2 h to 1 – 2 % of total Cu. The photosynthetic yield of *C. reinhardtii* strongly decreased after exposure for 1 or 2 h to dissolved Cu(II) in the concentration range 0.1 – 10 µM, whereas this decrease occurred in the total Cu concentration range 1 – 100 µM after exposure to CuNP. Effects of CuNP were compared to those of dissolved Cu(II) on the basis of dissolution experiments. CuNP effects on photosynthetic yield were similar or somewhat stronger for the same dissolved Cu^{2+} concentration. Addition of EDTA as a strong ligand for Cu(II) suppressed the toxicity of dissolved Cu(II) and of CuNP. These results thus indicate mostly effects of free Cu^{2+} to the algae.

* Corresponding author: laura.sigg@eawag.ch

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Introduction

Numerous nanoparticles have in recent years come into use for a variety of purposes and may be released into the environment [1] [2] [3]. Although copper nanoparticles are not listed under the most abundant nanomaterials in consumer products (Woodrow inventory), they are presently under development for various uses [4] [5]. Copper nanoparticles (CuNP) have properties of interest as conductive materials (e.g. as coatings, inks, pastes, electronic slurry for the miniaturization of microelectronic devices, lubricant additives), as catalysts for chemical reactions (US Research Nanomaterials, 2013), and as bactericides and fungicides. The use of copper nanoparticles may thus increase in the next years and lead to increased release of these NP into aquatic systems. Dissolution of copper nanoparticles to copper ions may occur under conditions of natural waters [6, 7].

Copper is well-known to be both an essential and a toxic element for aquatic organisms, with a strong dependence on its concentration and its speciation [8-10]. Copper interactions with both marine and freshwater algae have been extensively studied, e.g. [11-14]. Major conclusions of these studies have been that toxicity of copper to algae mostly depends on the free aquo copper ion concentration, which is regulated by complexation with natural organic matter or with other ligands [15]. In algae, copper may strongly affect photosynthesis [16, 17]. However, these studies have concerned the effects of dissolved Cu, whereas effects of CuNP have so far not been thoroughly investigated. Toxicity experiments have mostly been conducted with copper oxide nanoparticles [18-23] [24], whereas toxicity of elemental copper NP has only been examined in few cases [25]. Effects of CuNP on mammalian and fish cell lines have been attributed to a combination of effects of Cu ions and of specific processes induced by CuNP, e.g. production of ROS [25].

Toxicity of metallic nanoparticles to algae and to other organisms has been extensively investigated in the case of silver NP (AgNP) and of CeO_2-NP [26, 27]. The release of silver ions from AgNP has been shown in many cases to be the most important factor causing toxicity [26, 28-31]. In particular, there is no evidence of nanoparticle uptake in algae cells [26] [32]. In contrast, Ag^+ ions can readily enter cells over ion transport systems for essential ions, in particular for Cu [33]. It is thus of interest to consider if toxicity of CuNP is similar as AgNP and if their effects are mostly related to release of Cu^{2+} ions, which can easily enter the cells.

In this work, toxicity of carbon coated CuNPs to the unicellular green alga *Chlamydomonas reinhardtii* was investigated. The objectives of this work are to evaluate and compare the toxicity of CuNP with dissolved Cu^{2+} (provided as $CuSO_4$) and to link toxic effects of CuNP to their properties with respect to size distribution and dissolution. A crucial question is whether

the toxic effects of CuNPs originate only from Cu^{2+} ions or if there are some specific nanoparticle effects.

Materials and Methods

Chemicals

Copper nanoparticles (CuNP) were produced as carbon coated CuNP by the Particle Technology Laboratory at ETH Zurich, Switzerland using the flame-spray pyrolysis technique [34] [35]. CuNP were received in dry powder form. Two different CuNP batches were used for the experiments, referred to CuNP batch 1 and CuNP batch 2. CuNP have a spherical shape and a nominal size of 7.5 and 6 nm for batch 1 and 2, as determined by the producing laboratory. A stock solution with a nominal concentration of 1g L^{-1} total CuNP was produced in deionized nanopure water (18.2 MΩ cm at 25°C; Thermo Scientific Barnstead Nanopure, Skan AG, Basel-Allschwil, Switzerland) in a Teflon flask and ultrasonicated for 35 minutes. A new stock solution was prepared prior to each experiment.

Copper sulfate ($CuSO_4$), 3-morpholinopropane-1-sulfonic acid (MOPS), EDTA (etylenediaminetetraacetate), $CaCl_2$, nitric acid (HNO_3 65% suprapure) and NaOH were obtained from Sigma-Aldrich Corporation.

Nanoparticle characterization

The nanoparticle stock solution was diluted in MOPS (MOPS buffer (0.01 M) at pH 7.5). The size of nanoparticles in the exposure medium was determined by dynamic light scattering (DLS) using a Zeta-sizer (Nano ZS, Malvern Instruments) and by nanoparticle tracking analysis (NTA) using a NanoSight LM10 instrument. Using DLS, Z-average size and zeta potential were measured at four concentrations for batch 1 and at one concentration for batch 2 of CuNP over time for 2 hours. Measurements with high multimodal fit error and poor data quality according to warning from the Zeta-sizer instrument were excluded from further data evaluation. Two concentrations each were used for the NTA measurements.
Dissolution experiments were carried out at three different CuNP concentrations (2 mg L^{-1}, 8 mg L^{-1} and 30 mg L^{-1}) in the MOPS buffer medium over 2 hours. Dissolved Cu was separated from the nanoparticles by ultrafiltration (Amicon Ultra Tubes, containing regenerated Cellulose Centrifugal Filters with MWCO: 3000 Da). The UF-Tubes were then centrifuged for 30 min at 4000 rpm. The filtrates were analysed by ICP-MS after acidification and dilution. Dissolved Cu was also determined in a medium after exposure to algae.

Algal culture. The unicellular green alga *Chlamydomonas reinhardtii* was cultured in the growth medium Talaquil [36] in a Multitron incubation unit (Infors-HAT AG; Bottmingen,

Switzerland) with constant conditions of temperature (25°C), light (276 µE m^{-2} s^{-1}) and rotatory agitation (90 min^{-1}). Algae in the exponential growth phase were centrifuged (10 min at 3000 min^{-1}) and used for experiments.

Exposure of algae to CuNP and to dissolved Cu(II). All exposure experiments were carried out in a simple medium containing only MOPS buffer (0.01 M) at pH 7.5 to avoid complexation of Cu(II). This simple medium is only suitable for the algae for short-term experiments of few hours, as described in [37]. The experiments were carried out for both CuNP and $CuSO_4$ and at two different algae cell densities (low: 0.85 × 10^5 cells mL^{-1}; high: 10^6 cells mL^{-1}), with most experiments at the lower algae cell density. The CuNP concentrations were in the range 0.1 – 30 mg L^{-1}, and $CuSO_4$ concentrations in the range 0.1 – 30 µM. The experiments were carried out with 3 culture replicates.

Effects of CuNP and of dissolved Cu(II) on photosynthesis. The photosynthetic yield of Photosystem II of *Chlamydomonas reinhardtii* suspensions was measured with a pulse-amplitude modulation (PHYTO-PAM) instrument. The measured PSII yield was expressed as percentage of the control. For each concentration the average and standard deviation was computed from three experimental replicates. These values were plotted as concentration-response curves, which were then fitted to four parameter logistic regression curves. EC_{50} was calculated according to these fitted curves (SigmaPlot, 2013).

To differentiate between effects of ionic Cu^{2+} and nanoparticle effects, EDTA was added as a ligand for Cu^{2+} in the concentration range 1 – 100 µM. Preliminary experiments indicated that addition of Ca^{2+} together with EDTA was necessary to avoid effects on photosynthetic yield. $CaCl_2$ was thus added in excess of EDTA in these experiments. Photosynthetic yield was measured in these various treatments. Speciation calculations were carried out using the Vminteq program [38].

Results

Nanoparticle characterization

The Cu content of the CuNP (batch 2) was determined as 50 ± 12 %. The experimental Cu concentrations were calculated based on 50 % Cu and 50 % carbon. For the batch 1 CuNP the Cu content was assumed as 65%, as indicated by the NP producing laboratory. All experimental results and working concentrations in this work are related to the calculated Cu mass of the CuNP.

Many CuNP samples were not suitable for DLS measurements because of polydisperse size distribution and the presence of large particles. The number size distribution of the CuNP (batch 1) for 4 different CuNP concentrations (Figure 1) shows that the most abundant particle size was in the range of 200 nm. The size distribution was more uniform at higher CuNP concentration with narrower peaks. The smallest particles were indicated to be about 30 nm for a CuNP concentration of 1.3 mg L^{-1}, whereas large particles in the size range of 500 nm – >1000 nm were present in all samples.

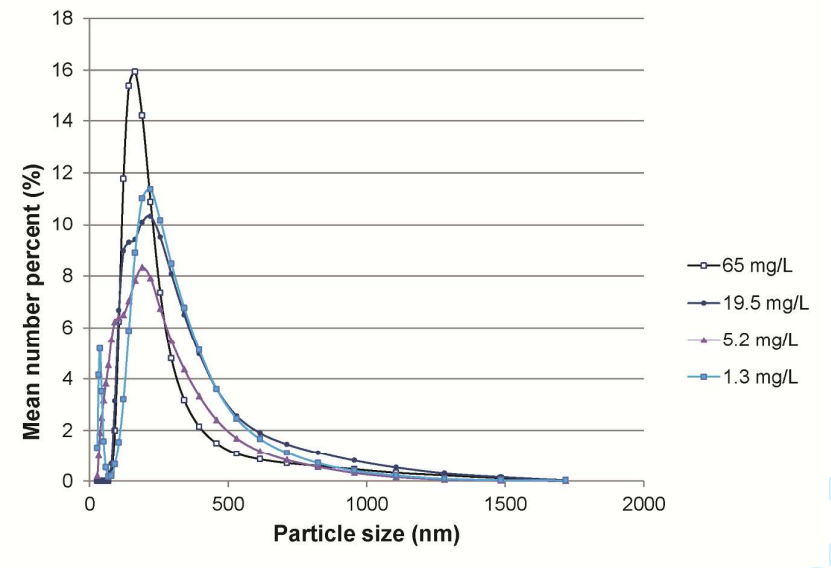

Figure 1: Size distribution of CuNP (batch 1) based on particle numbers at four concentrations in MOPS buffer (0.01 M, pH 7.5), measured by DLS.

The Z-average size of the CuNP was increasing with time over 1 hour to sizes around 400 – 500 nm, although the zeta potential was still negative around – 30 mV at the higher concentrations (SI figure 1).

The size of CuNP (batch 2) was examined both by DLS and NTA, by which a comparable size distribution was obtained with a mean size of 206 nm by Nanosight and a Z-average size of 488 nm by DLS, but a lower mode size of 142 nm (most frequent number of particles) by DLS (SI figure 2). The size differences between these two techniques are in relation to the known bias of DLS towards larger particle sizes, in particular if using the Z-average size.

The dissolved Cu concentration at the three tested CuNP concentrations increased with time over 2 h for both CuNP batches (Figure 2). After 2 hours, the dissolved concentration was about 3 times higher compared to the measured dissolved concentration at the start of the experiment. The dissolved fraction was higher in the case of CuNP (batch 1) with 0.5 – 2 % dissolved Cu (% of total Cu) than for CuNP (batch 2) with 0.4 – 0.8 % after 2 h, based on measured total Cu.

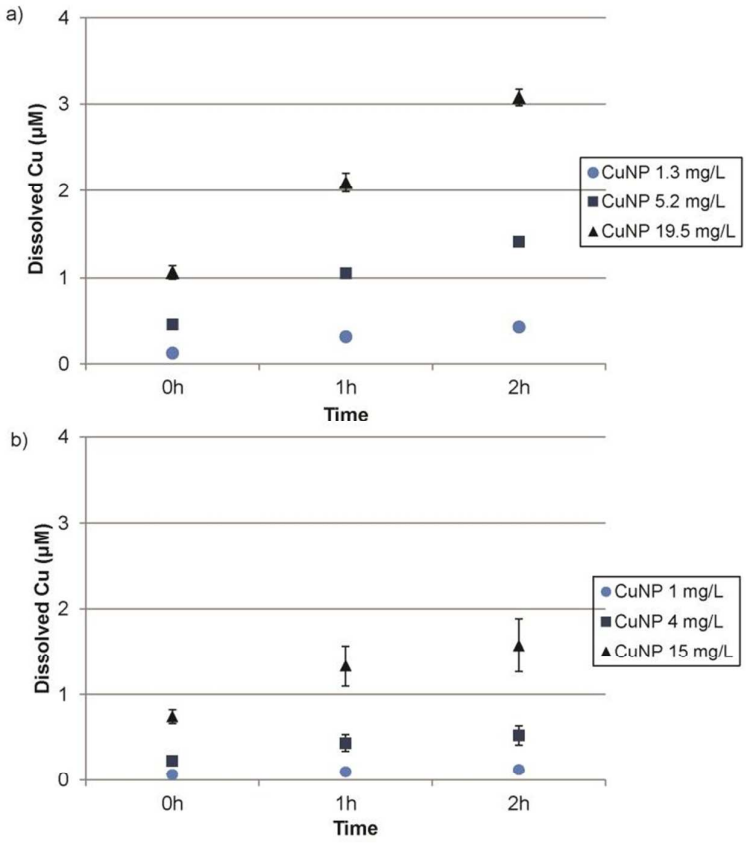

Figure 2. Dissolved Cu as a function of time over 2 h in CuNP suspensions in 0.01 M MOPS (pH 7.5) at three different concentrations of CuNP batch 1 (a) and batch 2 (b).

Effects of CuNP and of dissolved Cu(II) on photosynthetic yield

Photosynthetic yield as a function of total Cu concentration at the lower algal cell density (0.85×10^5 cells mL^{-1}) is presented in Figure 3 for dissolved Cu ($CuSO_4$) and the two CuNP batches. In the case of dissolved Cu the photosynthetic yield strongly decreased in the concentration range 0.1 – 10 µM, whereas this decrease occurred in the range 1 – 100 µM for

CuNP. The photosynthetic yield was not completely inhibited at CuNP concentrations > 100 µM.

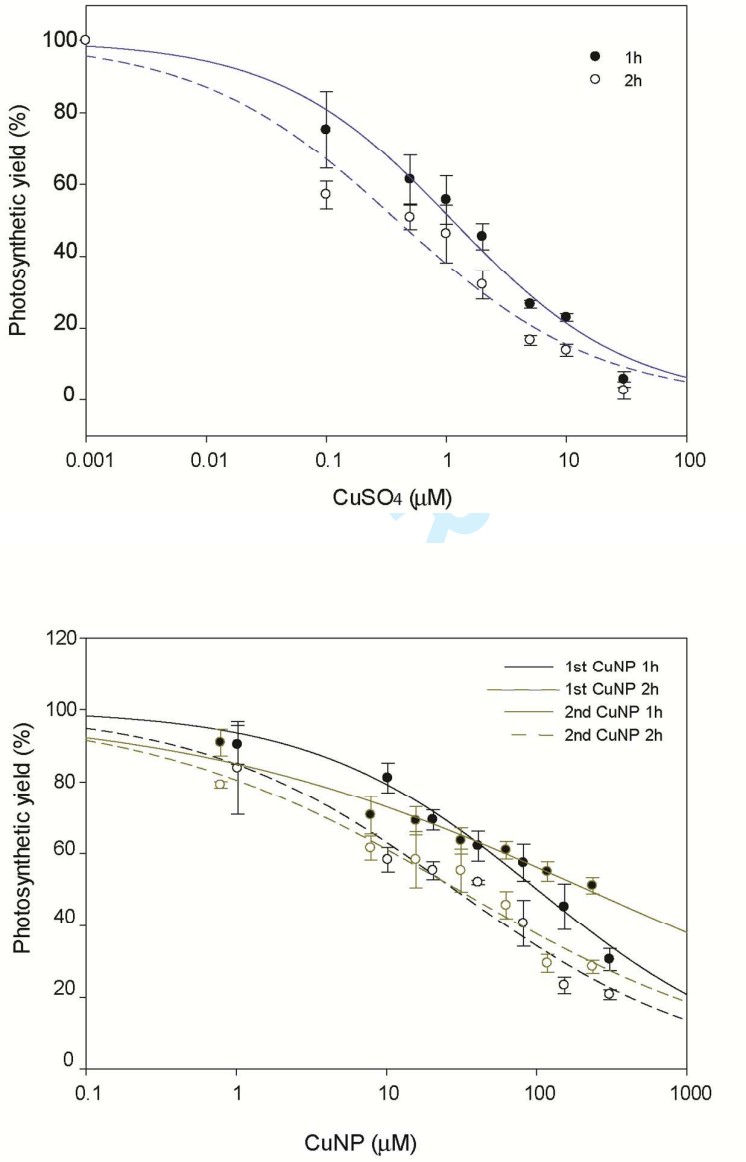

Figure 3. Photosynthetic yield after 1 h or 2 h as a function of total Cu concentration for dissolved Cu ($CuSO_4$) (a) and the two CuNP batches (b). Measured points and fitted regression curves used for EC_{50} calculations.

The calculated EC50 for photosynthesis inhibition as total Cu were thus much higher for CuNP than for dissolved Cu (Table 1). EC50 for CuNP (batch 1) was lower than for CuNP (batch 2) after 1 h, but after 2 h EC50 were similar for both batches.

Table 1. EC50 for photosynthesis inhibition by Cu^{2+} and CuNP (expressed as total Cu).

	EC50 1 h (μM Cu)	EC50 2 h (μM Cu)
Cu^{2+}	1.12 ± 0.16	0.39 ± 0.11
CuNP (batch 1)	101 ± 11	29 ± 5
CuNP (batch 2)	219 ± 55	30 ± 6

The effect of algal cell density was investigated at two different Cu^{2+} concentrations (1 and 10 μM) and at two CuNP concentrations (1 and 15 mg L^{-1}) (SI Figure 3). Exposure to 1 μM $CuSO_4$ led to a larger decrease in photosynthetic yield in low algal cell density samples compared to high algal cell density, for both 1 and 2 h. However, exposure to 10 μM $CuSO_4$ showed no difference in the used algae cell density, with photosynthetic yield values of about 23 % after 1 h and 13 % after 2 h of exposure at both high and low algae cell densities. In a similar way, effect of CuNP (1 mg L^{-1}) on photosynthesis was stronger in the case of low algal cell density than for high algal cell density, whereas a smaller difference occurred at the higher CuNP concentration (15 mg L^{-1}). These effects may be due to uptake of Cu by algae, which would decrease available Cu to a larger extent at high algal cell density and at lower Cu concentration. Measured dissolved Cu after contact with algae was about 30% lower than total Cu at both algal cell densities and indicated thus higher accumulation at the lower cell density (SI Figure S3).

Effect of CuNP on photosynthetic yield was compared with dissolved Cu(II) by relating the photosynthetic yield to the corresponding measured dissolved Cu(II) concentration (Figure 4). For the CuNP, the dissolved Cu^{2+} concentration was obtained from the dissolution experiments, using the average dissolved concentration from 0 to 1 h or from 0 to 2 h, respectively. In this way, it is taken into account that CuNP dissolution is a time dependent process and algae are therefore not exposed to the same Cu^{2+} concentration during the experiment. The photosynthetic yield obtained with CuNP was similar or somewhat lower for the same dissolved Cu^{2+}, with some lower values at high CuNP concentrations.

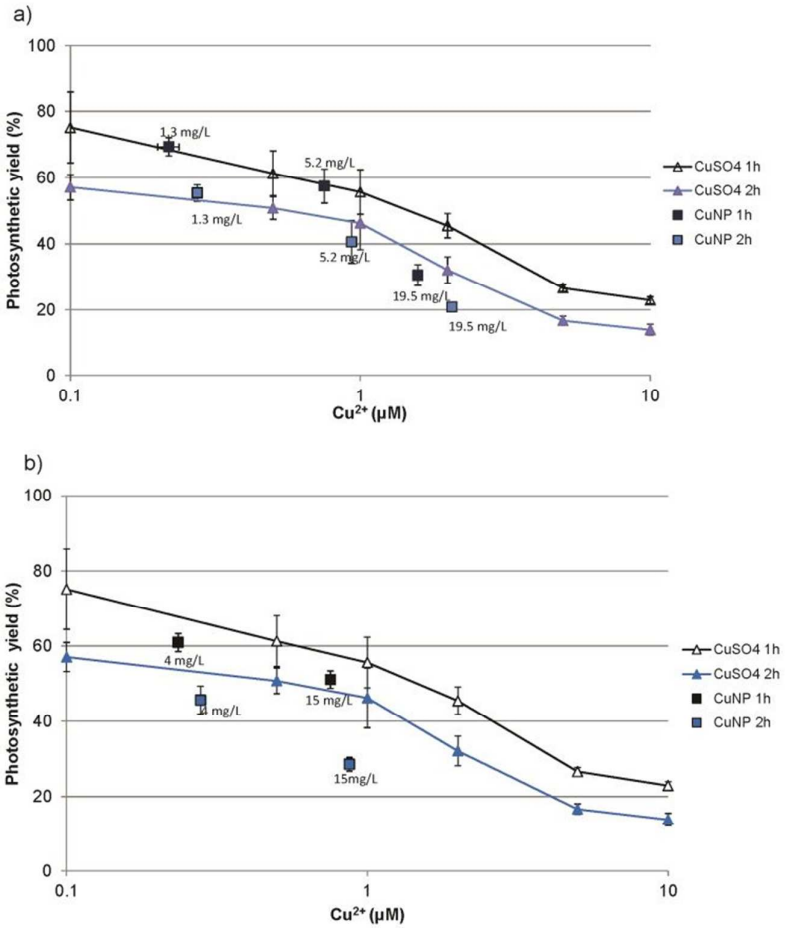

Figure 4: Photosynthetic yield as a function of dissolved Cu for $CuSO_4$ and CuNP (based on dissolution experiments), a) batch 1, b) batch 2.

Addition of EDTA to complex Cu^{2+} ions was first tested with dissolved Cu(II) to examine the effects on photosynthesis (Figure 5). The addition of EDTA alone in the absence of Cu(II) resulted in decreased photosynthetic yield, whereas the combined addition of Ca and EDTA had no effect. The combined addition of 10 µM EDTA and 10 or 20 µM Ca^{2+} to a solution containing 1 µM Cu suppressed the effect of Cu(II) on photosynthesis. In this case with 20 µM Ca^{2+}, both very low free Cu^{2+} and free EDTA are obtained under these conditions (Table S1 in SI). The addition of 10 µM EDTA and 20 µM Ca^{2+} to a CuNP suspension (15 mg L^{-1}; 1.5 µM dissolved Cu) nearly completely suppressed the toxic effects to photosynthesis after 2 h in the case of the high cell density experiment, but only partly in the case of the low cell density experiment (Figure 6). Similar results were obtained after 1 h.

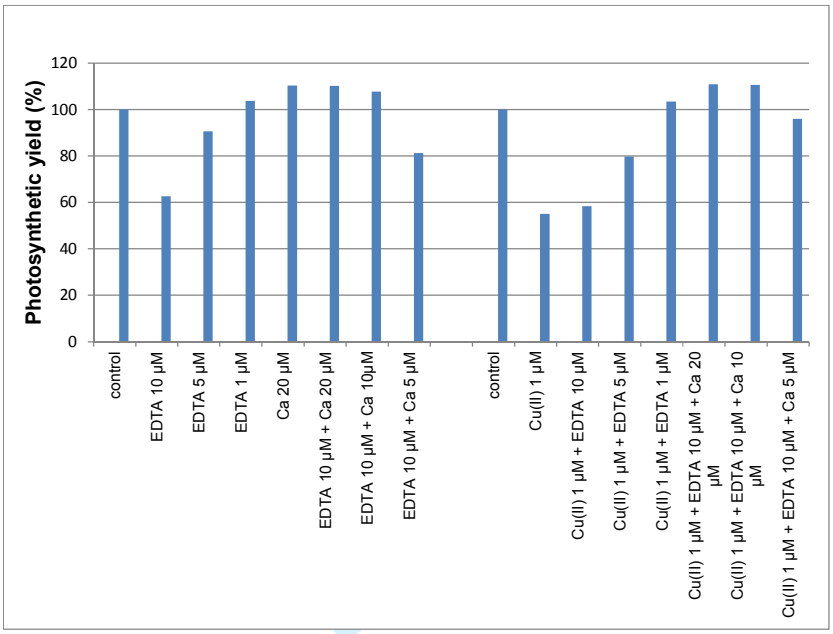

Figure 5: Photosynthetic yield after EDTA and combined EDTA + $CaCl_2$ addition to control algae and to algae treated with 1 µM Cu^{2+}.

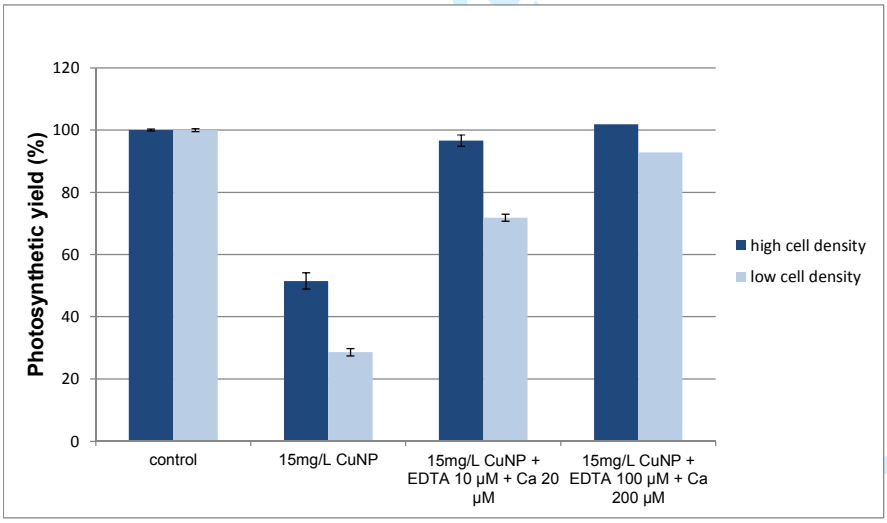

Figure 6: Photosynthetic yield of algae treated with 15 mg L^{-1} CuNP and with combined EDTA + $CaCl_2$ addition after 2 h exposure at two cell densities.

Discussion

Although the original size of the CuNP was indicated as 6 – 7 nm, the measured size of these CuNP in suspension at pH around 7.5 was always much higher, with average size of 140 – 200 nm and further size increase over few hours. A strong agglomeration of these CuNP is thus taking place within short time in suspensions at neutral pH, although the measured zeta-potentials are negative, in the range –10 – –30 mV. Agglomeration is in this case likely due to the hydrophobic properties of the carbon coating, which lead to limited stability in aqueous

suspension. In a similar way, these carbon-coated CuNP have been shown to strongly agglomerate in synthetic media, as well as under natural water conditions [6, 39]. Despite this rapid agglomeration, dissolution to Cu^{2+} is detectable after 2 hours, as shown in Figure 2, and continues over days, as shown in [6, 39]. The increased particle size does not prevent dissolution, and the carbon coating does also not completely protect the CuNP from dissolution and does not appear to be an efficient scavenger of dissolved Cu^{2+}.

Based on these findings about agglomeration and dissolution, the effects of nanoparticles have to be considered in comparison to effects of dissolved Cu^{2+}. The comparison of EC50 for photosynthesis in Table 1 indicates a much lower toxicity to photosynthesis of the CuNP in comparison to dissolved Cu^{2+}, based on total Cu. The direct comparison of photosynthesis yield as a function of dissolved Cu for CuNP and Cu^{2+} shows comparable results at low Cu concentrations and after only 1 h, but somewhat higher effects of CuNP at higher concentrations and longer exposure times (Figure 4). The large size agglomerates are very unlikely to be taken up in algae, although possible uptake of the much smaller original size nanoparticle should be more closely considered. Differences between CuNP and Cu^{2+} effects may be explained by either direct effects of CuNP or by increased dissolution of CuNP in contact with the algae. Direct effects of CuNP may include, in addition to unlikely uptake processes, shading effects leading to decreased light availability and sorption of CuNP to the algae surfaces. Shading effects have been shown to be of importance for the effects of carbon nanotubes on algae, at concentrations similar to those used here for CuNP [40]. However, the particle characteristics of CuNP are quite different from those of carbon nanotubes. In the case of agglomerated CeO_2-NP, no effects were observed up to high concentrations [32]. Sorption of CuNP to algae surfaces is a likely process, due to the hydrophobic characteristics of these NP, which favor interactions with organic compounds. It remains to be examined in more detail if sorption may lead to a substantial decrease of photosynthetic yield, or if dissolution of CuNP at close vicinity of the algae is the more efficient process. Dissolution of CuNP close to the cells is a possible process leading to increased Cu^{2+} in comparison to the dissolution experiments without cells, in a similar way as observed for AgNP [28] [41].

Further evidence for the role of dissolved Cu^{2+} was gained from the experiments involving EDTA addition. The speciation of EDTA appears to be of importance, as shown by the effects of EDTA alone in a simple buffer medium and the combined effects of Ca + EDTA, or Ca + EDTA + Cu. The addition of 10 µM EDTA alone to a MOPS buffer solution results in high concentrations of the EDTA species $HEDTA^{3-}$, H_2EDTA^{2-} and $EDTA^{4-}$, whereas if an equivalent concentration of Ca is added, most EDTA is bound to Ca. Free $HEDTA^{3-}$ and $EDTA^{4-}$ may damage the cell walls by binding Ca^{2+} and other cations involved in the cell wall structure [42, 43]. This effect is of minor importance in the presence of Ca. In the case of the combined addition of Ca + EDTA to a Cu solution, free Cu^{2+} is decreased to a very low concentration and free

EDTA is also kept low (Table S1). Toxicity of dissolved Cu^{2+} to photosynthesis was completely suppressed in this case (Figure 5). In the case of CuNP, the addition of EDTA + Ca also mostly suppressed the effects to photosynthetic yield (Figure 6). These findings indicate thus mostly effects of free Cu^{2+}.

These results for effects of CuNP to algae are in line with those obtained with AgNP, namely showing a predominant effect of dissolved Cu^{2+} or Ag^{+} ions, whereas some direct nanoparticle effects do not clearly appear [28, 41] [26]. However, in contrast to these results, interactions of CuNP with fish or mammalian cells indicated direct uptake and effects of CuNP [25]. Differences in nanoparticle uptake in fish and mammalian cells or in algae may lead to different pathways for effects of copper nanoparticles. Finally, the effects observed here may be related to copper concentrations in natural waters, which are usually in the range of 10 – 100 nM as dissolved Cu [44]. Effects of CuNP occur at quite high concentrations, which will likely only be present in highly polluted environments.

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Supplementary information

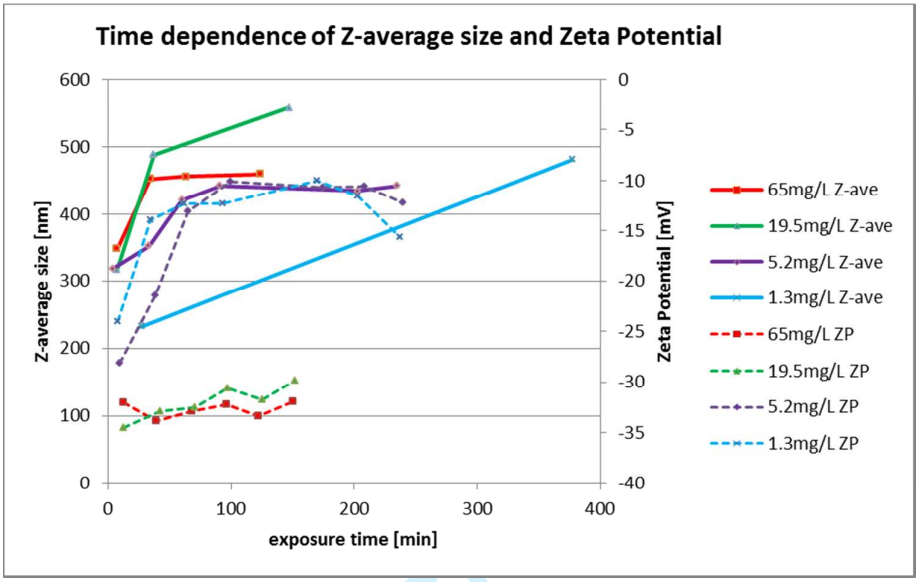

Figure S1: Time dependence of Z-average size and zeta potential at four different concentrations of CuNP (batch 1).

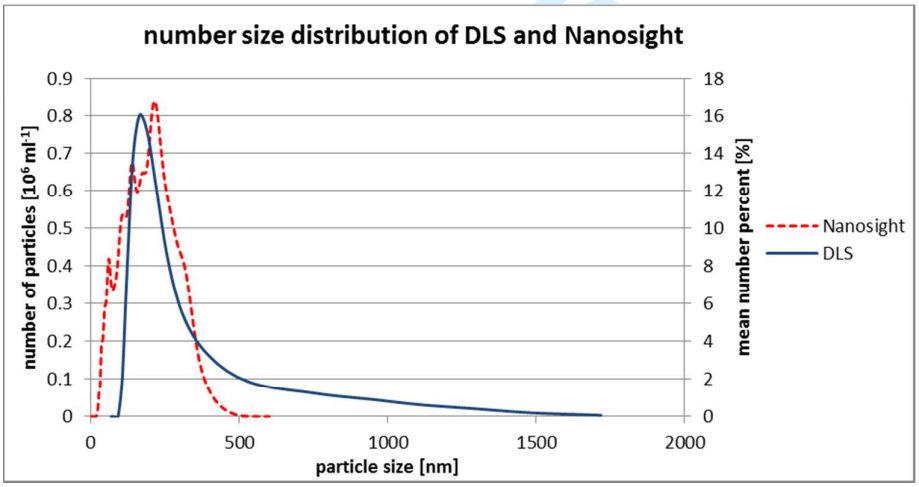

Figure S2: Size distribution of CuNP (batch 2) by DLS and NTA.

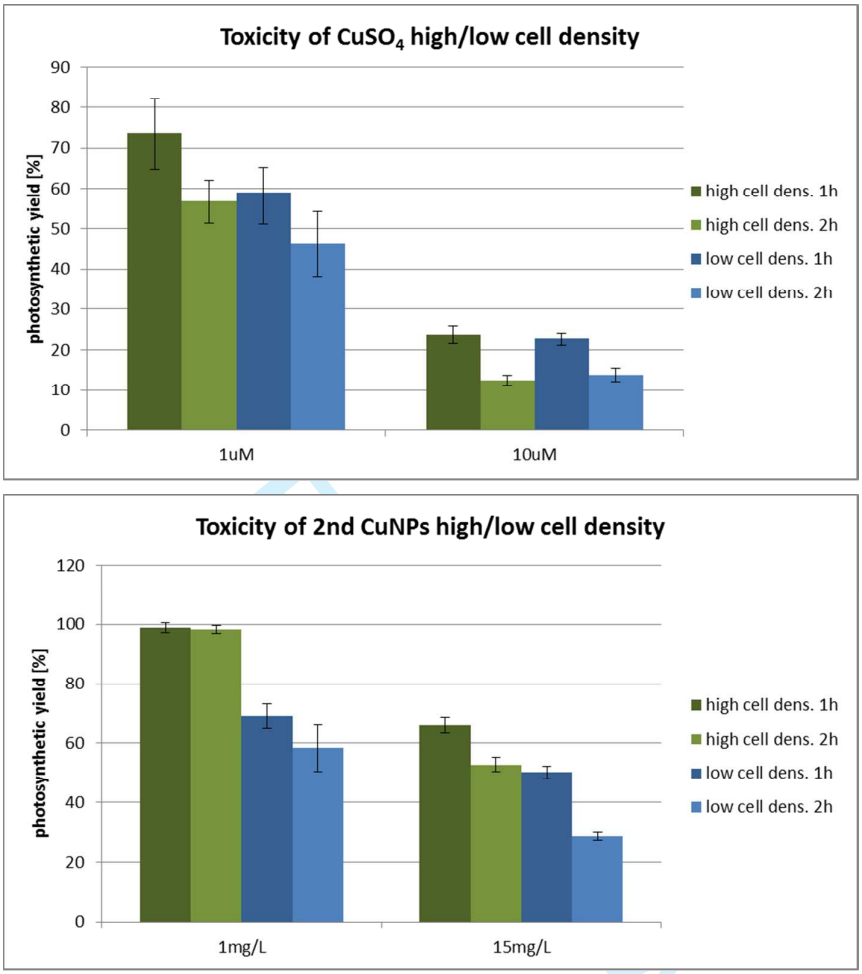

Figure S3: Effects of dissolved Cu(II) (a) and of CuNP (batch 2) (b) on photosynthetic yield of C. reinhardtii at two different cell densities (high cell density: $1x10^6$ cells mL^{-1}; low cell density: $0.85x10^5$ cells mL^{-1}).

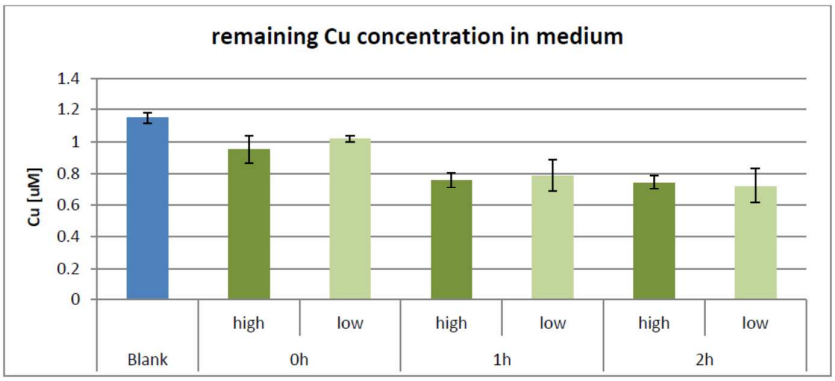

Fig. S4: Cu concentration in medium after algae exposure

Table S1. Speciation in presence of EDTA

With Ca^{2+} = 20 μM, EDTA = 10 μM, Cu = 1 μM, the calculated speciation of EDTA and Cu is:

Species	Concentration (M)
CuEDTA	0.99×10^{-6}
CaEDTA	8.99×10^{-6}
$HEDTA^{3-}$	2.1×10^{-9}
$EDTA^{4-}$	1.5×10^{-12}
Cu^{2+}	1.1×10^{-14}